

## REMARKS

### The Rejection Under 35 USC § 112, first paragraph

The Office Action alleges that claims 1,9,11, 12, 13, 15, 16, 37, 41-43, 46, 80 and 81 are not enabled.

The allegations however only appear to be directed to the method claims, while many of these claims, e.g., claims 37, 41-43, and 46, are compound claims. Clarification as to why the compound claims were rejected is respectfully requested.

The Office Action cites in view of Lemoine et al., in table 10.2 lists ras oncogene mutations in small-cell lung carcinoma (SCLC) as being of low-frequency. However, this does not mean to one of ordinary skill in the art that small-cell lung carcinoma cannot be treated by the ras pathway. Nothing in the reference states that such is the case.

Additionally, applicants attach a publication titled “Activated Raf-1 Causes Growth Arrests in Human Cell Lung Cancer Cells” which states on page 158, second column, that “data demonstrates that raf can function as a growth suppressor gene in SCLC.”

The issue still remains that not all cancers respond equally to affects on the ras/raf pathway. However, that is no basis for an enablement rejection. The Office Action admits that raf/ras pathway is correlated to many cancers, some highly, some intermediately, and so on. However, the law is clear that in cases, even where a broad claim includes inoperative embodiments, such is not problematic because one of ordinary skill in the art would know how to avoid the same. See *In re Dinh-Nguyen*, 181 USPQ 46 (CCPA 1974), and *In re Sarett*, 140 USPQ 474 (CCPA 1964).

This issue has also long been decided in *Sarett*, which states that

It is certainly not incumbent on an applicant who has made a broad ... invention and supported it by an adequately broad disclosure to demonstrate the operativeness of every substance falling within the scope of the broad claims to which he is entitled. ... The function of claims is to *point out* the invention and *define* the scope of the monopoly, not to exclude substances which are possibly of no use in practicing the invention. (Emphasis added.)

*Dinh-Nguyen*, states that

It is *not a function of the claims* to specifically exclude either possible inoperative substances or ineffective reactant proportions. (Emphasis added.)

In the present case, studies on the raf/ras pathway and related cancers thereto are numerous; and thus, one of ordinary skill in the art would know how to proceed in cases

where inoperative embodiments may occur. Applicants invented a broad invention, which under the applicable law, does not have to be limited to specific embodiments since the invention is not so limited.

Applicants submit that the claims are enabled, and that one of ordinary skill in the art in view of what is known in the art, can, without under experimentation, practice the claimed invention.

For all the foregoing reasons, reconsideration is respectfully requested.

Additionally, Lemoine et al., as admitted in the Office Action, teaches that pancreatic cancer, acute myeloid leukemia, and colorectal cancer are highly associated with the ras/raf kinase pathway, while bladder cancer is associated with the ras/raf kinase pathway to an intermediate extent. Consideration of some of the new claims in view of this admission is respectfully requested.

**The Rejection Under 35 USC § 112, second paragraph**

The Office Action alleges that “up to per-halosubstitution” is indefinite in the claims and that it is unclear what this range is encompassing and what the lower limit of this range is. Applicants respectfully disagree.

The language appears in claim 1, as “optionally substituted by one or more substituents independently selected from the group consisting of halogen, up to per-halosubstitution.” Since this is an optional substitution, it is clear that zero substitution is the lower limit. It is also clear that “up to per-...” means up to 4 substitutions. Thus, one of ordinary skill in the art would understand this language to mean 0, 1, 2, 3 or 4 halogen substituents. Changing the language to expressly state “0, 1, 2, 3 or 4 halogen substituents” merely would recite an equally clear alternate way to state the same, but would not further clarify the already clear original language. Thus, applicants decline to amend at this point.

In claims 37, 43 and 81 the substitution is not optional; thus, one of ordinary skill in the art would understand the language to mean “1, 2, 3 or 4 halogen substituents,” especially in light of the groups modified by the term are also separately recited in their non-substituted form.

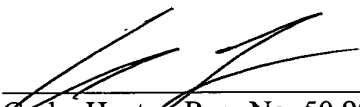
Reconsideration of this rejection is respectfully requested.

**The Specification**

The specification is amended to include the proviso from original claim 1.

The Commissioner is hereby authorized to charge any fees associated with this response or credit any overpayment to Deposit Account No. 13-3402.

Respectfully submitted,



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# Activated Raf-1 Causes Growth Arrest in Human Small Cell Lung Cancer Cells

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## Abstract

Small cell lung cancer (SCLC) accounts for 25% of all lung cancers, and is almost uniformly fatal. Unlike other lung cancers, ras mutations have not been reported in SCLC, suggesting that activation of ras-associated signal transduction pathways such as the raf-MEK mitogen-activated protein kinases (MAPK) are associated with biological consequences that are unique from other cancers. The biological effects of raf activation in small cell lung cancer cells was determined by transfecting NCI-H209 or NCI-H510 SCLC cells with a gene encoding a fusion protein consisting of an oncogenic form of human Raf-1 and the hormone binding domain of the estrogen receptor ( $\Delta$ Raf-1:ER), which can be activated with estradiol.  $\Delta$ Raf-1:ER activation resulted in phosphorylation of MAPK. Activation of this pathway caused a dramatic loss of soft agar cloning ability, suppression of growth capacity, associated with cell accumulation in G1 and G2, and S phase depletion. Raf activation in these SCLC cells was accompanied by a marked induction of the cyclin-dependent kinase (cdk) inhibitor p27<sup>kip1</sup>, and a decrease in cdk2 protein kinase activities. Each of these events can be inhibited by pretreatment with the MEK inhibitor PD098059. These data demonstrate that MAPK activation by  $\Delta$ Raf-1:ER can activate growth inhibitory pathways leading to cell cycle arrest. These data suggest that raf/MEK/MAPK pathway activation, rather than inhibition, may be a therapeutic target in SCLC and other neuroendocrine tumors. (*J. Clin. Invest.* 1998, 101:153–159.) **Key words:** SCLC • activated raf • MAP kinase • cell cycle • p27<sup>kip1</sup> • MEK inhibitor PD098059

## Introduction

c-raf-1 is a cytosolic serine/threonine protein kinase that is central to several intracellular signal transduction pathways. Activated ras can translocate raf to the cell membrane where it is activated by a process possibly involving tyrosine phosphoryla-

tion (1–4). Upon activation, c-raf-1 phosphorylates MEK, activating downstream mitogen-activated protein kinases (MAPK/ERKs). This phosphorylation cascade leads to the activation of transcription factors involved in cell growth and differentiation (5). Raf and mutated forms of ras act as dominant oncogenes, and can collaborate with other oncogenes to transform primary cells. In human lung cancers, activating ras mutations are common in adenocarcinomas and squamous cell cancers, but are not observed in small cell lung cancer (SCLC) (6). We previously showed that insertion of a mutated *ras* gene could differentiate some SCLC cell lines (7). These observations taken together suggested that activation of MAPK by ras mutation or raf activation might have growth suppressive activity in SCLC. We show here that activation of an estradiol-regulated form of human c-raf-1 (8) in SCLC cells can suppress growth by causing SCLC cells to become blocked in G1 and G2 of the cell cycle, unlike in other systems where raf activation results in transformation. This cell cycle arrest is associated with induction of the cyclin-dependent kinase (cdk) inhibitor p27<sup>kip1</sup>, occurs irrespective of whether p53 is mutated. Our findings indicate that activation of the raf/MEK/MAPK pathway can cause SCLC cells to arrest in G1 and G2, accompanied by p27<sup>kip1</sup> induction.

## Methods

**Cell culture and cell lines.** NCI-H209 and NCI-H510 human SCLC cell lines (9) were cultured in RPMI-1640 medium without Phenol red, 9% FBS (Sigma Chemical Co., St. Louis MO), 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin sulfate (Gibco, Grand Island, NY) in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. Cells were infected with equal volumes of retroviral supernatant from PA317-producer cells transfected with a retroviral vector pLNCX containing the activatable  $\Delta$ Raf-1:ER fusion construct. Infection of SCLC cells was augmented by 2  $\mu$ g/ml polybrene (Sigma Chemical Co.) in the medium. After 48 h, the medium was replaced by selection medium containing 0.5 mg/ml of G418. Pooled cultures of G418-resistant cells were grown, total RNA was extracted and analyzed for the presence of  $\Delta$ Raf-1:ER by Northern blot analysis. SCLC cells expressing  $\Delta$ Raf-1:ER were treated with 1  $\mu$ M  $\beta$ -estradiol to activate the  $\Delta$ Raf-1:ER fusion molecule. Untransfected Parent cells, parental cells exposed to 1  $\mu$ M  $\beta$ -estradiol, and cells transduced for  $\Delta$ Raf-1:ER but unexposed to  $\beta$ -estradiol were used as controls. No effects of  $\Delta$ Raf-1:ER transduction in the absence of estradiol were observed.

**Soft-agar cloning assay.** Soft agar cloning assays were performed in 35-mm dishes over a bottom layer of 0.8% low-melting agarose in growth medium and  $1.5 \times 10^4$  cells were plated in growth media containing 0.4% (wt/vol) agarose in the presence or absence of 1  $\mu$ M estradiol. After 3 wk of incubation in a humidified atmosphere con-

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1. **Abbreviations used in this paper:** BrdU, bromodeoxyuridine; cdk, cyclin-dependent kinase; MAPK, mitogen-activated protein kinase; SCLC, small cell lung cancer.

taining 5% CO<sub>2</sub> at 37°C, colonies with > 30 cells were scored, and percent cloning efficiency was calculated.

**Cell cycle analysis.** Cells were washed with ice cold 0.2% BSA in PBS, suspended in sucrose/citrate buffer (10). Nuclei were prepared, stained with propidium iodide and analyzed by an EPICS 752 flow cytometer (Coulter Electronics, Hialeah, FL) with a gate that selects single nuclei within a normal size range. The cell cycle parameters from 10,000 gated nuclei were determined by multicycle software (Phoenix Flow Systems, San Diego, CA). In some experiments, cells were pulse labeled with 1  $\mu$ M bromodeoxyuridine (BrdU; Sigma Chemical Co.) for 45 min at 37°C and were fixed in 70% ethanol/PBS. Extracted nuclei were stained with FITC-labeled anti-BrdU antibodies (Becton Dickinson, San Jose, CA) and propidium iodide. Flow cytometry analysis was performed as described above.

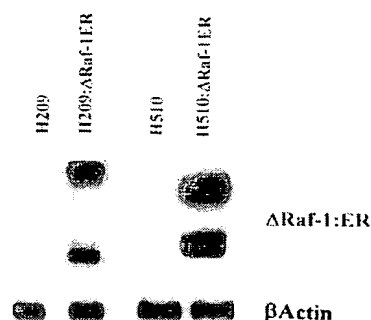
**Northern blotting.** Total RNA was extracted with an acid phenol-guanidinium isothiocyanate method (11). Total RNA (20  $\mu$ g/lane) was separated on 1.2% agarose/2.2 M formaldehyde-denaturing gels and transferred to Zeta-Probe (Bio-Rad, Melville, NY). Probes used in Northern analysis were  $\Delta$ Raf-1:ER, ClaI-XhoI fragment of pLNCX  $\Delta$ Raf-1:ER (8); and human  $\beta$ -actin, BamHI fragment (kindly provided by Don Cleveland, Johns Hopkins University, Baltimore, MD). These probes were labeled with [ $\alpha$ -<sup>32</sup>P] dCTP (Dupont, New England Nuclear, Boston, MA) by random primer labeling (Boehringer Mannheim, Indianapolis, IN). Hybridizations were done using radiolabeled probe (1  $\times$  10<sup>6</sup> cpm/ml) at 42°C for 16–18 h, then rinsed twice at room temperature and washed once for 30 min at 65°C with 1  $\times$  SSC and 1% SDS. Membranes were then exposed to x-ray film (Kodak X-0 MAT; Eastman Kodak Co., Rochester, NY) at –80°C with intensifying screens.

**Western blotting.** Cells were lysed in PBS, 1% Triton X-100, 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml pepstatin, and 2.5 mM PMSF. After protein concentrations were determined, 100  $\mu$ g of proteins were resolved on SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were probed with anti-Raf-1 (C-12), anti-p27<sup>Kip1</sup> (C-19), anticyclin E (HE12), anti-cdk2 (M2), anti-p34<sup>cdc2</sup> (C-17) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA); anti-MAPK (phosphospecific MAPK from New England Biolabs, Beverly, MA). Immunoreactive protein complexes were detected by enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL).

**Kinase assays.** Whole cell lysates were prepared in 50 mM Tris, pH 7.5, 137 mM NaCl, 5 mM MgCl<sub>2</sub>, 1% Triton X-100, 50 mM  $\beta$ -glycerophosphate, 2 mM EDTA, 10 mM EGTA, 1 mM DTT, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml pepstatin, and 1 mM phenylmethyl sulfonyl fluoride. 100  $\mu$ g of whole cell lysates were incubated for 2 h with 1  $\mu$ g/ml anti-cdk2 or anti-MAPK. The immune complexes were immunoprecipitated with protein A Sepharose beads (Pharmacia Fine Chemicals, Piscataway, NJ) for 1 h, and the immune complexes bound to the beads were washed with the same lysis buffer. Kinase assays were performed using histone H1 or myelin basic protein (MBP) (Sigma Chemical Co.), for cdk2 or MAPK, respectively. For kinase assays, the immunoprecipitates and substrates were incubated in a volume of 40  $\mu$ l containing 50 mM Tris (pH 7.5), 10 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mM DTT, 0.125  $\mu$ Ci/ $\mu$ l  $\gamma$ -<sup>32</sup>P ATP at 30°C for 20 min. Substrates for these kinase assays were either 20  $\mu$ g histone H1 for cdk2 assays, or 20  $\mu$ g myelin basic protein for MAP kinase assays. The reactions were stopped by the addition of 40  $\mu$ l of 2 $\times$  Laemmli buffer, boiled for 3 min and resolved on 12.5% SDS-PAGE gels. Phosphorylated proteins were visualized by autoradiography and quantitated on a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

## Results

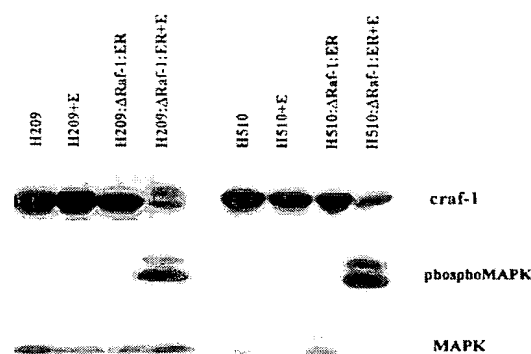
SCLC cells from cell lines NCI-H209 and NCI-H510 were transduced with a vector expressing  $\Delta$ Raf-1:ER. This vector encodes a conditionally activatable human c-Raf-1 which is transforming in many cell systems (8), and can differentiate some cells



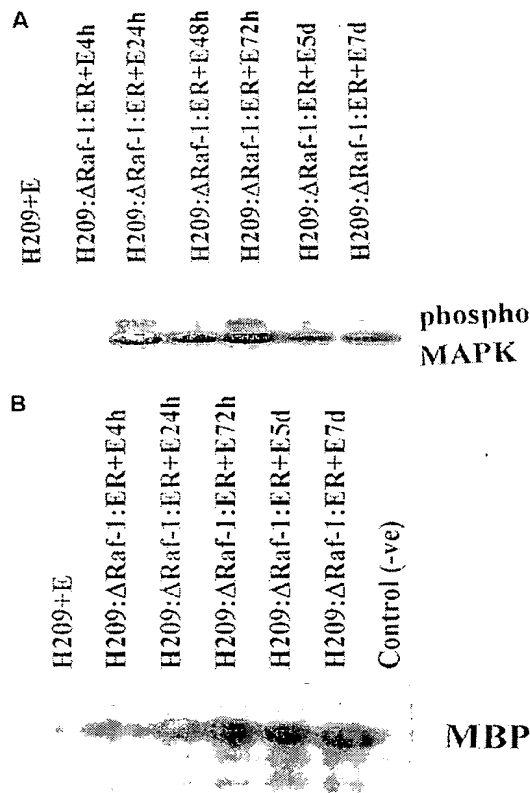
**Figure 1.** Northern analysis of  $\Delta$ Raf-1:ER expression in SCLC cells. Expression of  $\Delta$ Raf-1:ER after the stable transfection of NCI-H209 and NCI-H510 cells with a retroviral vector pLNCX containing the activatable  $\Delta$ Raf-1:ER fusion construct. Total RNA was extracted and 20  $\mu$ g of RNA was electrophoresed through a

1.2% formaldehyde gel and transferred to a nylon membrane. Northern analyses was performed as described in Methods and expression of  $\beta$ -actin served as loading control.

(12, 13).  $\Delta$ Raf-1:ER can be activated by the addition of 1  $\mu$ M estradiol or 4-hydroxy-tamoxifen to cells that stably express this construct. Pooled proliferating G418-resistant cultures of each transduced SCLC cells were analyzed for the expression of  $\Delta$ Raf-1:ER construct (Fig. 1). SCLC cells transfected with  $\Delta$ Raf-1:ER were exposed to estradiol and their  $\Delta$ Raf-1:ER-transduced counterparts without estradiol, as well as parental cells with and without estradiol, were used as controls. Activation of  $\Delta$ Raf-1:ER resulted in the phosphorylation of endogenous Raf-1 and downstream MAPK in NCI-H209 $\Delta$ Raf-1:ER and NCI-H510 $\Delta$ Raf-1:ER cells (Fig. 2). To measure the duration of MAPK activation, we activated  $\Delta$ Raf-1:ER for different periods of time and assessed the phosphorylation and enzymatic activity of MAPK. Phosphorylation of MAPK was similar in time periods from 24 h to 7 d after estradiol treatment; at the 4-h time point, phosphorylation of MAPK was very low (Fig. 3 A). Enzymatic activity of MAP kinase was also the same during this time period (Fig. 3 B). Estradiol activation of  $\Delta$ Raf-1:ER resulted in markedly changed cellular morphology



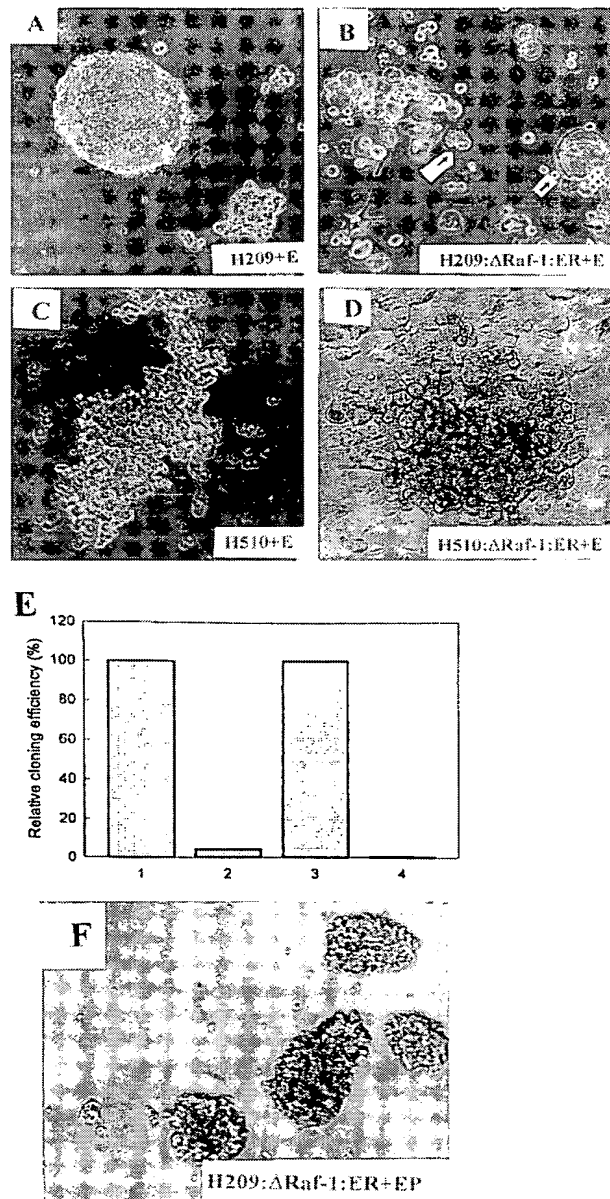
**Figure 2.** Activation of  $\Delta$ Raf-1:ER caused the phosphorylation of endogenous c-Raf-1 and MAP kinase in SCLC cells. Lysates from parental SCLC cells, SCLC cells treated with estradiol (+E) for 48 h, NCI-H209-transduced SCLC cells and estradiol-activated  $\Delta$ Raf-1:ER SCLC cells were immunoblotted with antibodies to Raf-1, phosphorylated MAPK and control MAPK.



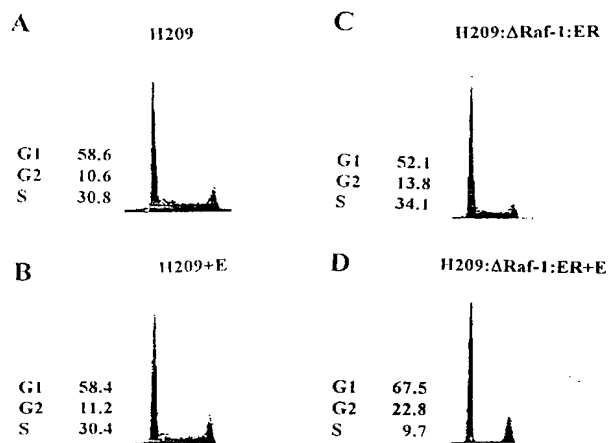
**Figure 3.** Phosphorylation and enzymatic activity of MAP kinase in  $\Delta$ Raf-1:ER-activated SCLC cells. (A) Lysates from parental NCI-H209 cells and NCI-H209: $\Delta$ Raf-1:ER cells with activated  $\Delta$ Raf-1:ER for different time periods were immunoblotted with phosphospecific MAPK antibody. (B) Whole cell lysates were immunoprecipitated with anti-MAPK and kinase activity was measured using myelin basic protein as a substrate as described in Methods. Phosphorylation and enzymatic activity of MAP kinase remained the same from 24 h to 7 d after  $\Delta$ Raf-1:ER activation.

in NCI-H209: $\Delta$ Raf-1:ER cells and NCI-H510: $\Delta$ Raf-1:ER cells. Upon estradiol activation of  $\Delta$ Raf-1:ER, these cells, which normally grow in suspension as floating suspended cell clusters (9), adhered to the plastic substratum of the tissue culture flasks, formed multinucleated giant cells, proliferated poorly, and virtually lost the ability to grow in soft agar (Fig. 4). This was not an effect of the endogenous estrogen receptor, since  $\Delta$ Raf-1:ER activation by 4-hydroxy-tamoxifen (4-HT) in NCI-H209: $\Delta$ Raf-1:ER and NCI-H510: $\Delta$ Raf-1:ER cells had similar effects on growth, morphology, and cloning efficiency (data not shown). No effect of estradiol was observed on parental cells. We observed similar effects of  $\Delta$ Raf-1:ER activation in NCI-

**Figure 4.** Morphological effects of activated  $\Delta$ Raf-1:ER in NCI-H209 and NCI-H510 SCLC cells. Cells were grown in the presence of 1  $\mu$ M estradiol (+E) for 6 d. Photographs of parental cells and transduced cells were taken using phase-contrast light microscope at a magnifica-



tion of 100. No morphological changes were observed in parental cells grown in the absence or presence of estradiol. Parental cells exposed to estradiol (A and C) were used as controls for  $\Delta$ Raf-1:ER maintain morphological characteristics as floating clusters of cells as do  $\Delta$ Raf-1:ER-transduced cells without added estradiol. Activation of  $\Delta$ Raf-1:ER caused the floating, suspended clusters of parental cells to become surface adherent and form multinucleated giant cells (B and D). (E) Activated  $\Delta$ Raf-1:ER inhibited soft agar cloning. Soft agar cloning of NCI-H209(1), NCI-H209: $\Delta$ Raf-1:ER(2), NCI-H510(3), and NCI-H510: $\Delta$ Raf-1:ER(4) cells in the presence of 1  $\mu$ M estradiol. (F) PD098059 blocked the morphological changes induced by activation of  $\Delta$ Raf-1:ER. NCI-H209: $\Delta$ Raf-1:ER cells were treated with 10  $\mu$ M PD098059 for 45 min before to the addition of 1  $\mu$ M estradiol and grown for 72 h in the presence of PD098059 and estradiol (+EP) and photographed at a magnification of 100.



**Figure 5.** Effect of activated  $\Delta$ Raf-1:ER on cell cycle distribution. SCLC NCI-H209 cells and their transduced  $\Delta$ Raf-1:ER cells (A and C) were exposed to 1  $\mu$ M estradiol (+E) for 6 d (B and D) and harvested for cell cycle distribution analysis using propidium iodide. Histograms show relative DNA content on x-axis and number of nuclei on the y-axis. Cell cycle distribution is similar to NCI-H209 and NCI-H510 cells after  $\Delta$ Raf-1:ER activation.

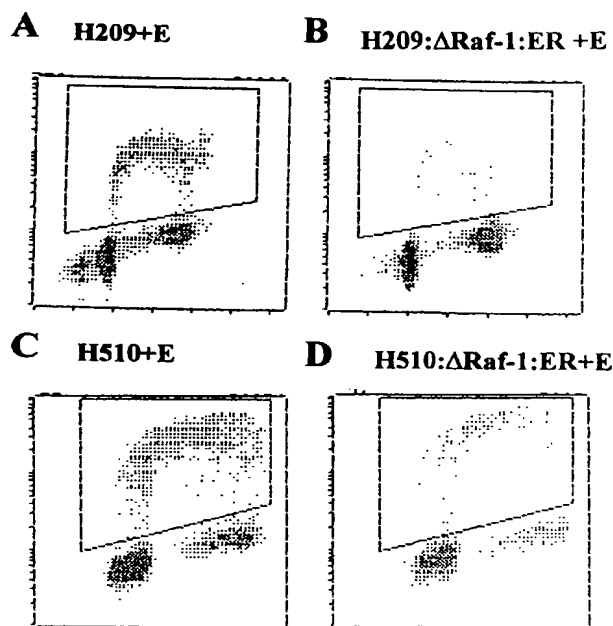
H345, NCI-H69, and DMS53 SCLC cells (data not shown). The proportion of apoptotic cells ( $\sim 5\%$ ) was not increased by  $\Delta$ Raf-1:ER activation (data not shown), suggesting that the raf-induced growth suppression was due to inhibition of cell cycle progression.

Cell cycle analyses using propidium iodide and bromodeoxyuridine demonstrated cell cycle specific growth arrest upon  $\Delta$ Raf-1:ER activation.  $\Delta$ Raf-1:ER activation reduced the proportion of NCI-H209: $\Delta$ Raf-1:ER and NCI-H510: $\Delta$ Raf-1:ER cells in S phase and caused the cells to accumulate in both G1 and G2 (Fig. 5). No effects on cell cycle kinetics were observed in control NCI-H209 and NCI-H510 cells treated with estradiol, nor in  $\Delta$ Raf-1:ER-transduced cells in the absence of estradiol. Cell cycle progression from G1 to S, measured by BrdU incorporation, was decreased by 79% in NCI-H209 cells, and by 77.3% in NCI-H510 cells upon  $\Delta$ Raf-1:ER activation (Fig. 6). Karyotypic analysis failed to detect any metaphases in estradiol treated NCI-H209: $\Delta$ Raf-1:ER and NCI-H510: $\Delta$ Raf-1:ER cells (unpublished results).

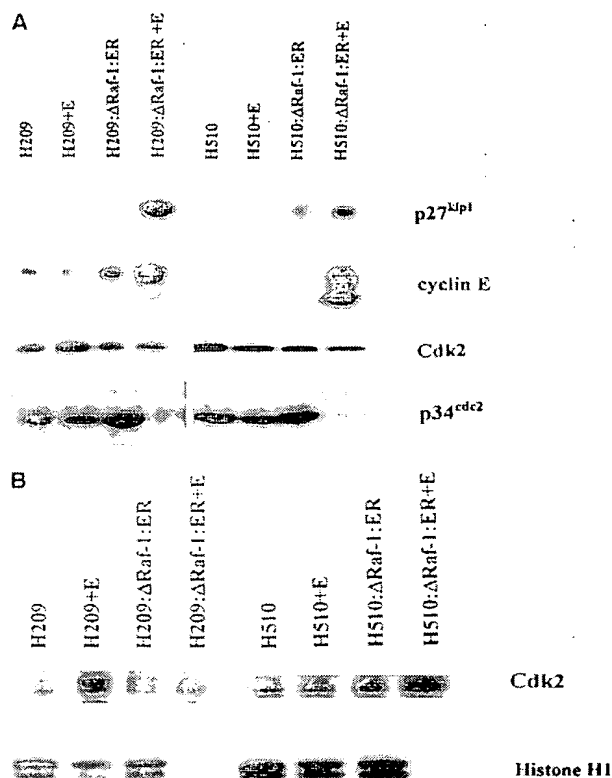
Since cyclins and their cdk2s regulate the G1/S and G2/M transitions in the mammalian cell cycle (14), we examined whether cyclin-cdk complexes are altered in the cell cycle blocked SCLC cells after  $\Delta$ Raf-1:ER activation. Members of the cyclin D family act in mid G1 by complexing with either cdk4 or cdk6 (15, 16), while cyclin E acts in late G1 by complexing with cdk2 (17, 18). As with most SCLC cells, NCI-H209 cells and NCI-H510 cells both lack a functional retinoblastoma susceptibility gene (Rb) (19, 20); p53 is mutated in NCI-H510 cells, and is wild-type in NCI-H209 cells (21). In pRb-defective cells, transition from G1/S is regulated by cyclinE-cdk2 complexes, but not by cyclin D-cdk4 or cyclin D-cdk6 (15, 16). Previous work has shown that cyclin D family members are poorly expressed in SCLC cells (22). Consistent with this observation, we were unable to detect cyclins D1, D2,

or D3 in NCI-H209 and NCI-H510 cells with or without activated  $\Delta$ Raf-1:ER. Cyclin E, however, was actually induced after  $\Delta$ Raf-1:ER activation over the same time interval that NCI-H510 and NCI-H209 cells undergo cell cycle arrest (Fig. 7A). Analysis of cdk2 expression by Western blotting demonstrated that neither activation of  $\Delta$ Raf-1:ER nor treatment of the parental NCI-H209 and NCI-H510 cells with estradiol had any influence on cdk2 protein levels. Although cyclin E and cdk2 protein levels were not diminished in SCLC cells after  $\Delta$ Raf-1:ER activation, the enzymatic activity of cdk2 was markedly reduced. Activation of  $\Delta$ Raf-1:ER in SCLC cells reduced cdk2 kinase activity, as measured by histone H1 phosphorylation, by 91% in NCI-H209 cells and 78% in NCI-H510 cells (Fig. 7B). Since it has been shown previously that cyclin E-cdk2 activity is necessary for S phase progression (23), these data suggest that  $\Delta$ Raf-1:ER activation may block G1-S progression by reducing cdk2-associated kinase activity.

Decreased cyclin-cdk2 activity can result from expression of the cdk inhibitors p21<sup>Cip1/Waf1</sup> or p27<sup>Kip1</sup>, which bind cyclin-cdk complexes and inhibit their kinase activity. Overexpression of these cdk inhibitors has been shown to cause G1 arrest in several cell types (24). We were unable to detect p21<sup>Cip1/Waf1</sup> in NCI-H510: $\Delta$ Raf-1:ER cells or NCI-H209: $\Delta$ Raf-1:ER cells, whether or not  $\Delta$ Raf-1:ER was activated, nor by exposure of NCI-H510 and NCI-H209 cells to estradiol (data not shown). In contrast, p27<sup>Kip1</sup> protein was induced by  $\Delta$ Raf-1:ER activation in NCI-H510: $\Delta$ Raf-1:ER and NCI-H209: $\Delta$ Raf-1:ER



**Figure 6.** BrdU labeling of SCLC cells. SCLC control cells and their transduced  $\Delta$ Raf-1:ER cells were grown in the presence of estradiol (+E) and were pulsed with BrdU. BrdU incorporation was measured using FITC-labeled anti-BrdU. Bitmaps were set using FITC-unlabeled cells. Histograms representing the BrdU labeling (y-axis) vs. the DNA staining (x-axis) and percentage of BrdU incorporated cells in S phase were measured using 10,000 cells.



**Figure 7.** (A) Activation of  $\Delta$ Raf-1:ER induced the expression of cyclin E, cdk inhibitor p27<sup>kip1</sup>, reduced p34<sup>cdc2</sup> protein, and cdk2 kinase activity. Parental cells and their  $\Delta$ Raf-1:ER-expressing cells were grown in the presence (+E) or absence of 1  $\mu$ M estradiol for 6 d and harvested for proteins. Western blot analysis demonstrated that p27<sup>kip1</sup> is induced 20-fold in NCI-H209 cells and ninefold in NCI-H510 cells after  $\Delta$ Raf-1:ER activation, compared with control cells. Expression of cyclin E (50–52 kD) was also increased in cells expressing activated  $\Delta$ Raf-1:ER. In contrast,  $\Delta$ Raf-1:ER activation markedly reduced p34<sup>cdc2</sup> protein in these SCLC cells. No effects on cdk2 protein expression were observed after  $\Delta$ Raf-1:ER activation. (B) cdk2 activity immunoprecipitated from SCLC cells was measured using histone H1 as substrate. Activation of  $\Delta$ Raf-1:ER led to decreased cdk2 kinase activity in NCI-H209:ΔRaf-1:ER cells by 91 and 78% in NCI-H510:ΔRaf-1:ER cells, compared with their untreated control cells. cdk2 protein levels from cdk2 immunoprecipitates were also shown.

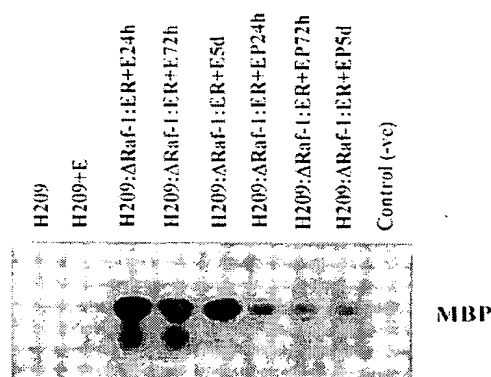
cells, but was not evident in NCI-H510 and NCI-H209 control cells with or without estradiol (Fig. 7A). Northern blot analyses showed that p27<sup>kip1</sup> mRNA levels were not altered after  $\Delta$ Raf-1:ER activation (data not shown), consistent with previous data that p27<sup>kip1</sup> can be posttranscriptionally regulated (25).

Since both NCI-H209:ΔRaf-1:ER and NCI-H510:ΔRaf-1:ER SCLC cells also arrested in G2 after  $\Delta$ Raf-1:ER activation, we examined whether mediators of the G2 transition were also modified in these cells. It has been shown that p34<sup>cdc2</sup> (cdc2) association with cyclin B to form a complex with kinase activity is essential for the G2/M transition (26), and that this

molecule is present at fairly constant levels throughout the cell cycle. Activation of  $\Delta$ Raf-1:ER in NCI-H209:ΔRaf-1:ER and NCI-H510:ΔRaf-1:ER cells strongly reduced p34<sup>cdc2</sup> protein expression (Fig. 7A), suggesting that the G2 block in these cells may be due to interference with cyclin B-cdc2 activity. Since p27<sup>kip1</sup> also inhibits p34<sup>cdc2</sup> activity (27, 28), our data suggest that p27<sup>kip1</sup> may contribute to both the G1 and G2 blocks we have observed.

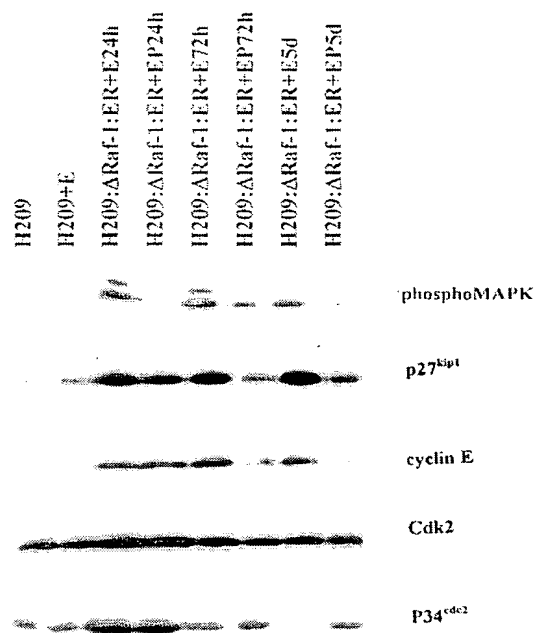
The  $\Delta$ Raf-1:ER-dependent cell cycle block we observed resulted from activation of the MEK–MAPK signal transduction pathway. To determine this, we treated NCI-H510 and NCI-H209 cells transduced with  $\Delta$ Raf-1:ER and controls with the MEK inhibitor, PD098059. PD098059 selectively blocks the activation of MEK, and thereby inhibits phosphorylation and activation of MAPK (Figs. 8 and 9). PD098059 allowed NCI-H209 and NCI-H510 cells with estradiol activated  $\Delta$ Raf-1:ER to proliferate, and abolished cell accumulation in G1 and G2 after  $\Delta$ Raf-1:ER activation. Further, PD098059 reversed the morphological changes induced by  $\Delta$ Raf-1:ER activation in SCLC cells (Fig. 4F). Similarly, PD098059 prevented the induction of cyclin E and p27<sup>kip1</sup>, and the reduced levels of p34<sup>cdc2</sup> that follow  $\Delta$ Raf-1:ER activation in SCLC cells (Fig. 9). Also our finding is in accordance with another study that MEK inhibitor PD098059 can inhibit the  $\Delta$ Raf-1:ER-induced p21<sup>cip1</sup> cdk inhibitor (31). There was no discernible effect of PD098059 on parental NCI-H209 and NCI-H510 cells (data not shown).

Our data indicate that  $\Delta$ Raf-1:ER-mediated activation of MAPK inhibits the growth of SCLC cells, causing these cells to accumulate in the G1 and G2 phase of the cell cycle. This appears to be due in part to the induction of the cdk inhibi-



**Figure 8.** PD098059 inhibited the phosphorylation of MAPK. NCI-H209:ΔRaf-1:ER cells were treated with 10  $\mu$ M PD098059 for 45 min before the addition of 1  $\mu$ M estradiol and grown for 24, 72 h, and 5 d in the presence of PD098059 and estradiol (+EP). PD098059-treated cell lysates and control cell lysates were immunoprecipitated with anti-MAPK and kinase assays were performed using MBP as substrate.  $\Delta$ Raf-1:ER-activated NCI-H209:ΔRaf-1:ER cell lysate was used for negative control and kinase assay was performed without primary antibody. PD098059 inhibited the phosphorylation of MBP by 60% in  $\Delta$ Raf-1:ER activated NCI-H209:ΔRaf-1:ER cells.





**Figure 9.** PD098059 inhibited the phosphorylation of MAPK, blocked the induction of p27<sup>kip1</sup>, cyclin E, and restored p34<sup>cdc2</sup> levels in  $\Delta$ Raf-1:ER-activated cells. NCI-H209: $\Delta$ Raf-1:ER cells were treated with 10  $\mu$ M PD098059 for 45 min before the addition of 1  $\mu$ M estradiol and grown for 5 d in the presence of PD098059 and estradiol (+EP). Western blots were made from the cell lysates of NCI-H209: $\Delta$ Raf-1:ER cells exposed to PD098059 and estradiol together (+EP) and estradiol alone (+E) for different time intervals and probed with phosphospecific MAPK antibody, anti-p27<sup>kip1</sup>, anti-cyclin E, anti-cdk2, and anti-p34<sup>cdc2</sup>, respectively.

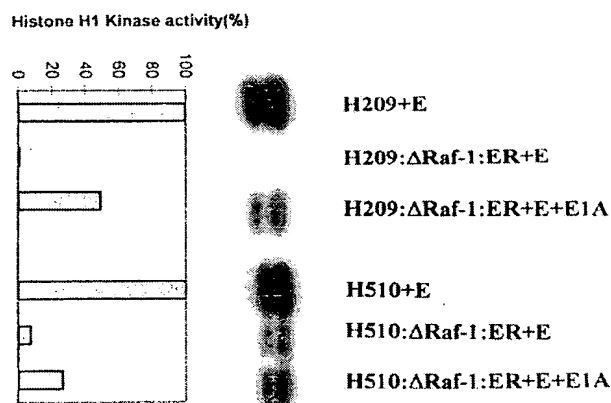
tor p27<sup>kip1</sup> and its inactivation of cdk2. We examined whether p27<sup>kip1</sup> was responsible for the inactivation of cdk2 by use of a p27<sup>kip1</sup> inhibitor in cell extracts. p27<sup>kip1</sup> can be inactivated by adenovirus protein E1A, resulting in restoration of cdk2 activity (32). We tested, therefore, whether addition of E1A to cell extracts from NCI-H209: $\Delta$ Raf-1:ER and NCI-H510: $\Delta$ Raf-1:ER, to inactivate the induced p27<sup>kip1</sup>, would restore cdk2 activity in these cell extracts. As shown in Fig. 10, E1A restored cdk2 activity to 48% of control levels in 209: $\Delta$ Raf-1:ER cells and to 26% of controls in 510: $\Delta$ Raf-1:ER cells. This indicates that p27<sup>kip1</sup> contributes significantly to the loss of cdk2 activity, and further suggests that p27<sup>kip1</sup> contributes significantly to the cell cycle block observed after  $\Delta$ Raf-1:ER activation.

## Discussion

The Raf/MEK/MAPK pathway has been shown to contribute to the transformation of primary cells and stimulation of cellular proliferation (1–3). In some cell types the Raf/MEK/MAPK pathway leads to cell differentiation (33, 12, 13). Our results show that  $\Delta$ Raf-1:ER activation inhibits the growth of SCLC cells and is associated with the accumulation of cells in the G1 and G2 phases of the cell cycle. Our data suggest that this cell

cycle block occurs via induction of the cdk inhibitor p27<sup>kip1</sup>, leading to reduced cdk2 activity. Previous studies have shown that p27<sup>kip1</sup> inhibits a wide array of cyclin–cdk complexes, and that overexpression of p27<sup>kip1</sup> causes many cells to arrest in G1 (27, 28). Our data are similar to those observed when TGF- $\beta$  induces cell cycle arrest in some systems (34, 35). However, addition of TGF- $\beta$  to NCI-H510 and NCI-H209 cells only modestly increased p27<sup>kip1</sup> levels, and was unable to influence cdk2 activity or modify the cell cycle (data not shown). The addition of conditioned medium from the activated  $\Delta$ Raf-1:ER cells to NCI-H209 and NCI-H510 parental cells also failed to influence the cell cycle (data not shown). These data indicate that the effects of  $\Delta$ Raf-1:ER activation and resultant MAPK phosphorylation in our SCLC cells are not dependent on autocrine production of TGF- $\beta$ .

These data demonstrate that raf can function as a growth suppressor gene in SCLC, a common neuroendocrine cancer, by activating signal transduction pathways coupled to regulatory molecules of the cell cycle. A role for the Ras/Raf/MAPK pathway in growth arrest or cellular differentiation has been suggested by earlier studies. Ectopic expression of v-Ras causes growth inhibition in Schwann cells and REF52 cells (36, 37). Human medullary thyroid carcinoma cells (12, 38), pheochromocytoma (PC12) cells (33), and hippocampal neuronal cells (13) are differentiated by ras and raf. In PC12 cells, ras-regulated hypophosphorylation of pRb mediates growth inhibition and neuronal differentiation (39). Also recent data have suggested that the ras pathway is capable of inducing premature cell senescence after the induction of cdk inhibitor p16<sup>INK4a</sup> (40). Our findings that activation of MAPK by  $\Delta$ Raf-1:ER causes SCLC cells to arrest in the cell cycle extend these concepts, and also indicate that MAPK can activate cdk inhibitors such as p27<sup>kip1</sup> that can function to reduce cdk2 activity. Our findings suggest consideration of members of the raf/MEK/MAP



**Figure 10.** E1A viral oncoprotein restored cdk2 activity in activated  $\Delta$ Raf-1:ER cells. 50 ng of an E1A 243 amino acid-purified protein (32), were added to 50  $\mu$ g of activated NCI-H209: $\Delta$ Raf-1:ER or NCI-H510: $\Delta$ Raf-1:ER cell extracts and incubated on ice for 30 min. cdk2 immunocomplexes were made and kinase activity was determined as described before. Histone H1 phosphorylation was quantitated on a Phosphorimager to calculate the percentage of restoration of cdk2 activity.

kinase pathway as therapeutic targets in the treatment and chemoprevention of SCLC and other neuroendocrine tumors.

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